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Abstract

There are currently no treatment methods on the market for modifying OA – only drugs that help relieve pain and inflammation but do not address the underlying disease, allowing it to continue destroying joint tissues. When joint function becomes severely impaired and other management strategies are ineffective, arthroscopic or joint replacement surgeries become the only options for patients. More than 50% of people worldwide who are over 65 years of age show radiological evidence of OA. These patients and younger populations who are susceptible to OA onset due to sports injuries or other factors could significantly benefit from the development of strategies to mitigate disease progression. Furthermore, post-traumatic OA experienced by military personnel injured on the battlefield is a highly accelerated process, likely because these combat injuries are complicated by factors that are associated with greater risk including bone loss, surrounding soft tissue damage, and infection. In these combat-related cases, post-traumatic OA typically manifests less than 2 years after injury compared to post-traumatic OA resulting from sports injuries that takes about 10 years to develop.

The goals of this work are to test the ability of a novel therapeutic to hinder the progression of post-traumatic osteoarthritis. This debilitating joint condition more severely affects military service personnel who have sustained injuries in combat resulting from high energy impacts such as explosions, fragment projectiles, and gunshot wounds. The therapeutic we propose is derived from human amniotic membrane, which has shown promise in clinical studies for various regenerative applications. The objectives of the work are to characterize two formulations of the injectable therapeutic, determine its capacity for treating OA in a small animal model, then scale up the approach to a clinically relevant loading and disease progression timeframe in a large animal model in order to outline a pathway to human clinical trials of the treatment method.

Our central hypotheses are that joint retention time and therapeutic efficacy will be influenced by amniotic membrane particle size, treatment timing, and frequency of delivery in well-established small and large animal models of post-traumatic OA. These hypotheses will be tested via three Specific Aims: *Aim 1:* Evaluate the effects of human amniotic membrane (AM) particle size distribution on particle retention and progression of OA after 3 weeks in the rat medial meniscal transection (MMT) model. *Aim 2:* Assess differences in therapeutic efficacy of single and multiple post-injury particle injection treatments on OA progression during a 6 week time period. *Aim 3:* Evaluate therapeutic effects in an established ovine unilateral medial meniscectomy (MM) model of OA.

Table of Contents

	Page
1. Introduction	4
2. Keywords	4
3. Overall Project Summary	4
4. Key Research Accomplishments	13
5. Conclusion	14
6. Publications, Abstracts, and Presentations	14
7. Inventions, Patents and Licenses	14
8. Reportable Outcomes	14
9. Other Achievements	14

1. INTRODUCTION:

Osteoarthritis (OA) is a debilitating articular joint condition resulting in functional impairment for nearly 27 million Americans at a cost of over $128 billion per year for the U.S. economy. Post-traumatic OA (PTOA) associated with combat extremity injuries has a 26% greater incidence in active military service personnel 20-24 years of age and over twice the incidence after age 40 compared to the general population. There are no disease modifying OA therapeutics currently approved. The goal of this work is to develop a safe and effective long-term strategy, using intra-articular delivery of micronized dehydrated amnion/chorion membrane (dHACM), to inhibit OA disease progression following trauma. Factors we hypothesized would impact therapeutic efficacy of dHACM included particle size, timing of treatment, and frequency of delivery. Thus, the study aims involved varying and more fully characterizing particle size distribution, injecting at different time points, and utilizing single or multiple injections, in both the rat medial meniscus transection (MMT) model and the sheep medial meniscectomy (MM) model.

2. KEYWORDS:

Osteoarthritis therapeutic	Intra-articular injection	Amnion/chorion membrane	Rat medial meniscal transection	Sheep medial meniscectomy
Particle size distribution	therapeutic timepoints	EPIC-µCT	Articular cartilage	Subchondral bone
Osteophytes	Proteoglycans			

3. OVERALL PROJECT SUMMARY:

In the second annual funding period (Sept 2015 – Sept 2016), significant progress was made in Specific Aims 1, 2, and 3; Major Tasks 1, 2, 3, 4, and 5.

***Specific Aim 1*:** Evaluate effects of micronized human amniotic membrane particle size distribution on particle retention and progression of OA after 3 weeks in the rat medial meniscal transection model

Major Task 1: Characterization of particle size distribution and retention.

Subtask 1: Characterize size distribution

- Size distribution measurements of two particle size distributions of dHACM have been performed using light microscopy and ImageJ analyses to identify and measure particle area.

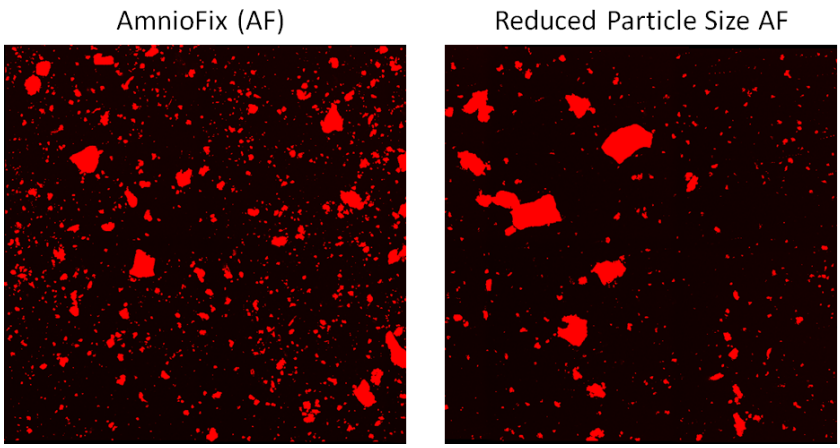

Figure 1. ImageJ representations of dHACM particles during edge detection and areal calculation algorithm image processing approaches. Standard micronized dHACM (AmnioFix or AF) and reduced particle size AmnioFix (RPS AF) are shown.

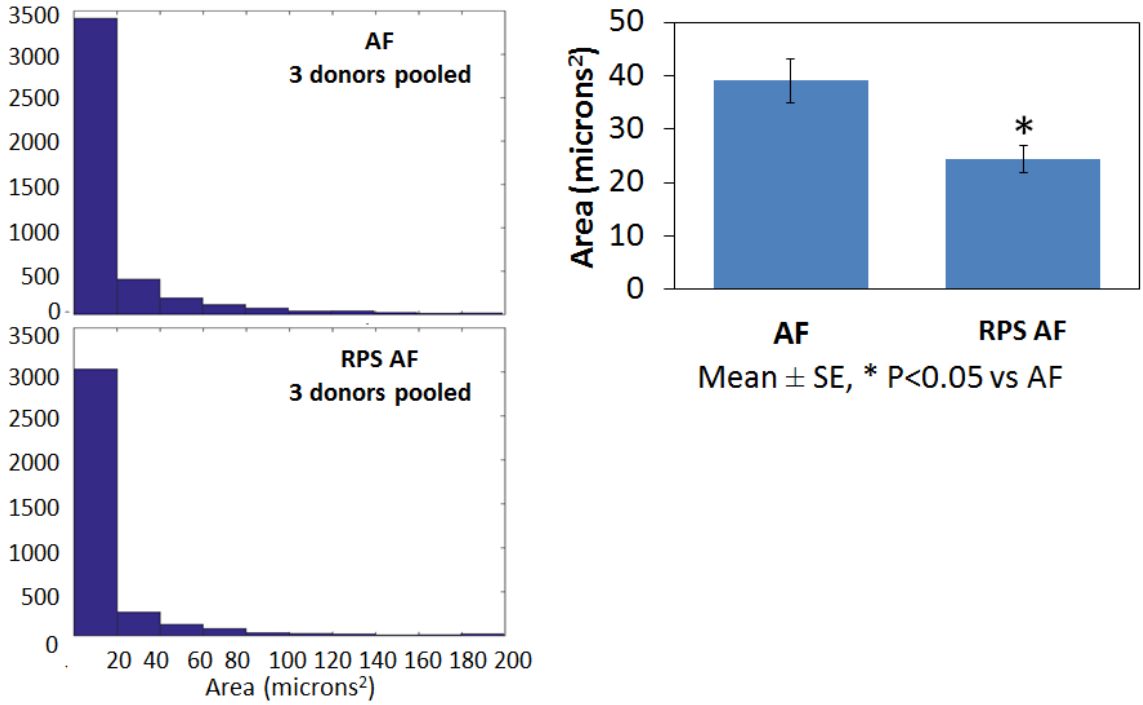

Figure 2. Quantification of particle size distribution of AmnioFix and RPS AmnioFix using areal measurements in 2D ImageJ analyses. Using a jackknife statistical estimation test of the sampling of areal measurements, the following results were determined: RPS AF had more particles < 10µm^2, AF had more particles 10-250µm^2, no significant difference was seen in the number of particles >250µm^2 for the two particle size distributions.

Subtask 2: Submit documents for IACUC approval (Completed and previously documented in Year 1 annual report)

Milestone #1: IACUC approval was received and previously documented in Year 1 annual report

Subtask 3: Particle preparation and surgical procedures

- VivoTag 680 XL and VivoTag-S 750 were explored as options for labeling dHACM. Preliminary testing of four label:dHACM ratios, two washing protocols, and four excitation/emission frequency combinations was conducted *in vitro* out to 14 days.
- Preliminary naïve rat knee injections were performed using dHACM labeled with both VivoTag 680 XL and VivoTag-S 750 (n=3 each). Fluorescent signal was measured over a time course of 24 days.

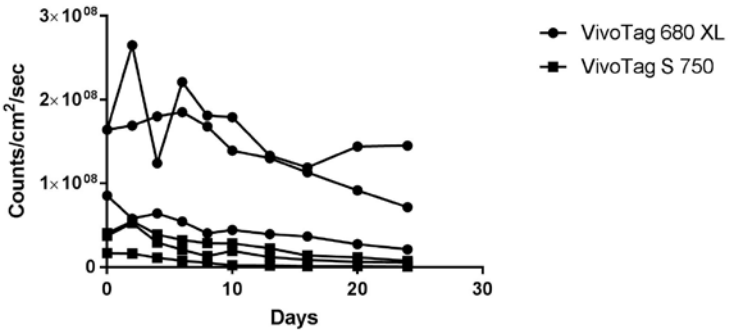

Figure 3. Fluorescence intensity measured in naïve rat knee joints after injection of dHACM labeled with VivoTag 680 XL and VivoTag-S 750 (Perkin Elmer) at 0, 2, 4, 6, 8, 10, 13, 17, 20, and 24 days (n=3 each).

- VivoTag 680 XL (Perkin Elmer, excitation 675 emission 720) at 1:20 dye:dHACM ratio was chosen for subsequent studies.
- Rats underwent MMT or sham surgeries in left knees, then were injected at 24hrs post-surgery with AmnioFix or Reduced Particle Size (RPS) AmnioFix labeled with VivoTag 680 XL (or saline control).

Subtask 4: Establish particle retention profiles for two size distributions.

- Animals were imaged at 1, 3, 5, 7, 9, 11, 14, 18, 21, 32, 35, 39, and 42 days after injection of labeled dHACM (IVIS Spectrum CT).

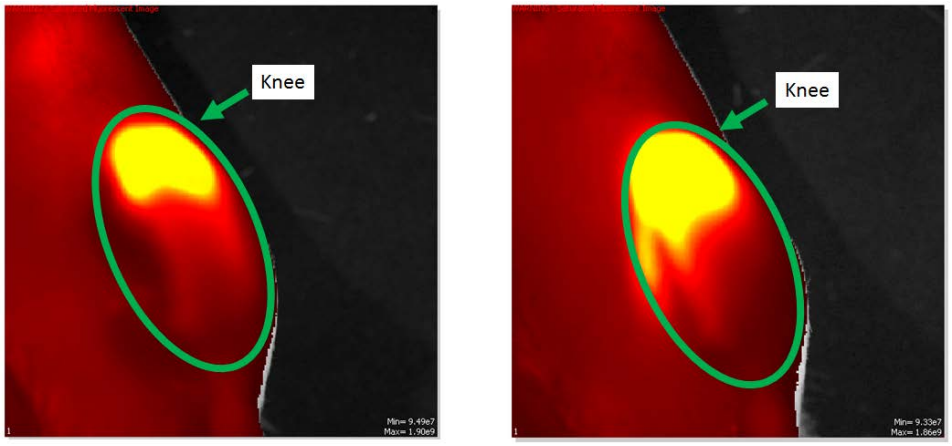

Figure 4. Representative fluorescent images of rat knees after injection of dHACM labeled with VivoTag 680 XL at 1:20 dye:dHACM ratio. Left = AmnioFix (micronized / traditional particle size distribution). Right = RPS AmnioFix (reduced particle size distribution).

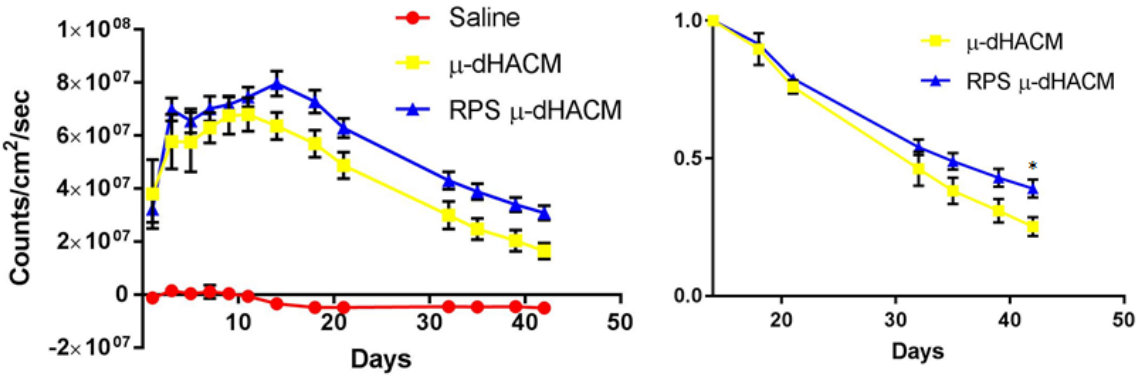

Figure 5. Quantification of fluorescence intensity at time points out to 42 days. Left = absolute measurements; right = normalized to Day 14 (n=4-5). There was no steady decrease in fluorescence signal until Day 14. At 42 days after injection, RPS AmnioFix was shown to have significantly higher normalized signal intensity. Quantification of saturated area demonstrated similar results (not shown).

- Ongoing work: Histological processing and sectioning of knee joint tissue containing labeled dHACM to determine whether particles can be identified and located in histology sections

Major Task 2: Assess 3 week therapeutic efficacy *in vivo* for two size distributions.

Subtask 1: Rat MMT surgical and treatment procedures for analyzing effectiveness of the two size distributions of dHACM after 3 weeks. (Completed and previously documented in Year 1 annual report)

Subtask 3: Evaluation of therapeutic effects using EPIC-μCT and histology (Completed and previously documented in Year 1 annual report)

Specific Aim 1 Conclusions

dHACM was successfully labeled with two fluorophores, and VivoTag 680 XL (Perkin Elmer) was chosen to perform further characterization of *in vivo* dHACM retention and localization in our studies of the rat MMT model of PTOA. Work is ongoing to more completely understand the clearance profiles and location of AmnioFix and RPS AmnioFix particles in the treatment of rat knee joint degeneration induced by MMT.

Previously documented in Year 1 annual report: Changes in articular cartilage and subchondral bone morphology and composition due to MMT surgery were able to be quantified at both 1 and 3 weeks after joint destabilization. Treatment with micronized dHACM (AmnioFix) demonstrated better preservation of cartilage proteoglycan (PG) content (inversely related to cartilage attenuation) at 1 and 3 weeks post-surgery. Reducing the particle size distribution did not further attenuate OA disease progression compared to the standard AmnioFix size distribution and resulted in increased cartilage thickness at 3 weeks. The majority of alterations to subchondral bone (density, thickness) were detected at 3 weeks (not at 1 week post-surgery) whereas cartilage attenuation and surface roughness differences were already seen at 1 week post-surgery. This suggests that in the rat MMT model, degenerative changes occur first in the articular cartilage and subchondral bone changes follow.

Milestone #2: A manuscript, including work summarized for Major Task 2, is in the final stages of preparation.

***Specific Aim 2*:** Assess differences in therapeutic efficacy of single and multiple post-injury particle injection treatments on OA progression during a 6 week time period.

Major Task 3: Assess therapeutic efficacy for single or multiple intra-articular injections.

Subtask 1: Surgical and treatment procedures. (Completed and previously documented in Year 1 annual report)

Subtask 3: Evaluation of therapeutic effects using EPIC-μCT and histology. (Completed and previously documented in Year 1 annual report)

Specific Aim 2 Conclusions

Previously documented in Year 1 annual report: Articular cartilage composition, subchondral bone, and osteophyte data showed a beneficial effect of single dHACM injection at 3 wks post-MMT surgery, suggesting that after disease progression has begun, dHACM can provide protection against degradative effects. However, a single injection of dHACM 24 hrs post-MMT surgery did not provide a protective effect out to 6 wks post-surgery. Multiple injection dHACM treatment (at 24 hrs + 3 wks post-MMT surgery) demonstrated histological results suggesting moderate inhibition of cartilage damage;

however, EPIC-µCT quantification showed somewhat inconclusive results that may require further investigation.

Milestone #3: A manuscript, including work summarized for Major Task 3, is in preparation.

***Specific Aim 3*:** Evaluate therapeutic effects in an established ovine unilateral medial meniscectomy model of OA

Major Task 4: Produce and characterize ovine unilateral medial meniscectomy OA model.

Subtask 1: Submit documents for IACUC/ACURO and CRADA approvals

- IACUC and CRADA documents for full study were submitted, required modifications, and have received final approval: CRADA 15-DEC-2015, IACUC 01-MAR-2016.

Subtask 2: Surgical procedures for ovine medial meniscectomy (MM). (Completed and previously documented in Year 1 annual report)

Subtask 3: Ovine model EPIC-µCT protocol development. (Completed and previously documented in Year 1 annual report)

Subtask 4: Ovine cartilage and subchondral bone characterization.

Knee joints from two 12wk post-surgery animals and one 8wk post-surgery animal have been evaluated previously. During this reporting period, three 24wk post-surgery stifle joints have been analyzed. Representative images from one of these animals are shown in Figure 6.

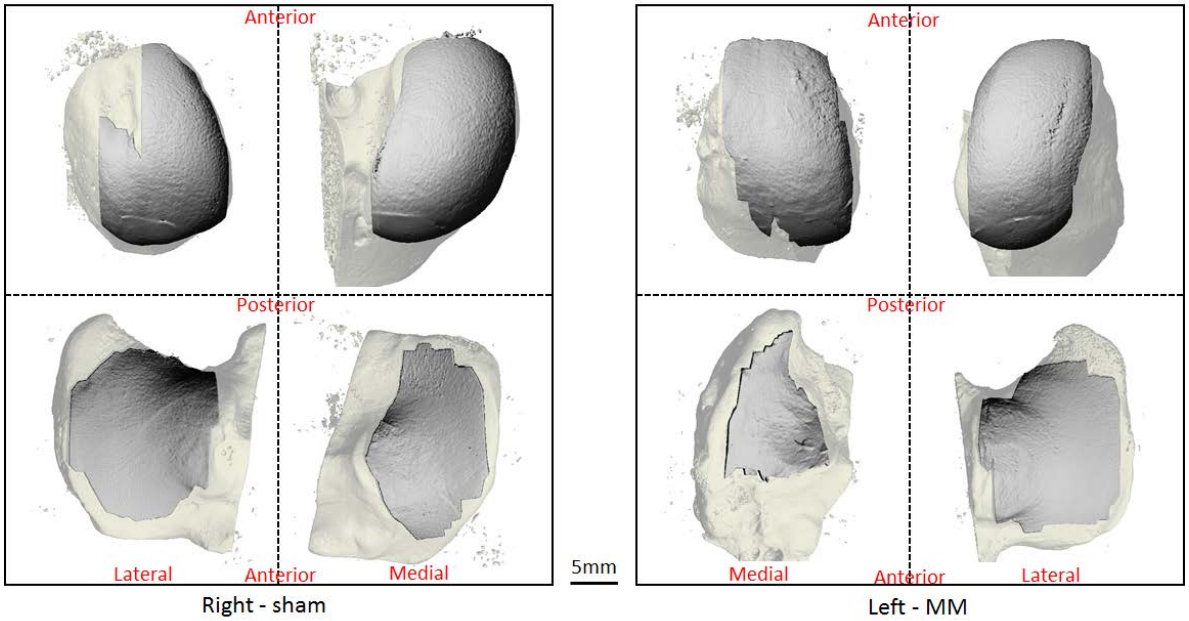

Figure 6. Example cartilage-bone overlay images for 24wk post-surgery femur and tibia after Hexabrix contrast-enhancement. Qualitative differences were seen in left medial compartments compared to right - articular cartilage not as smooth, visible areas of cartilage wear, cartilage margins more difficult to discern.

Furthermore, marginal bone remodeling was evident in 2D and 3D images (best depicted through 3D renderings of bone only segmentations, shown in Figure 7).

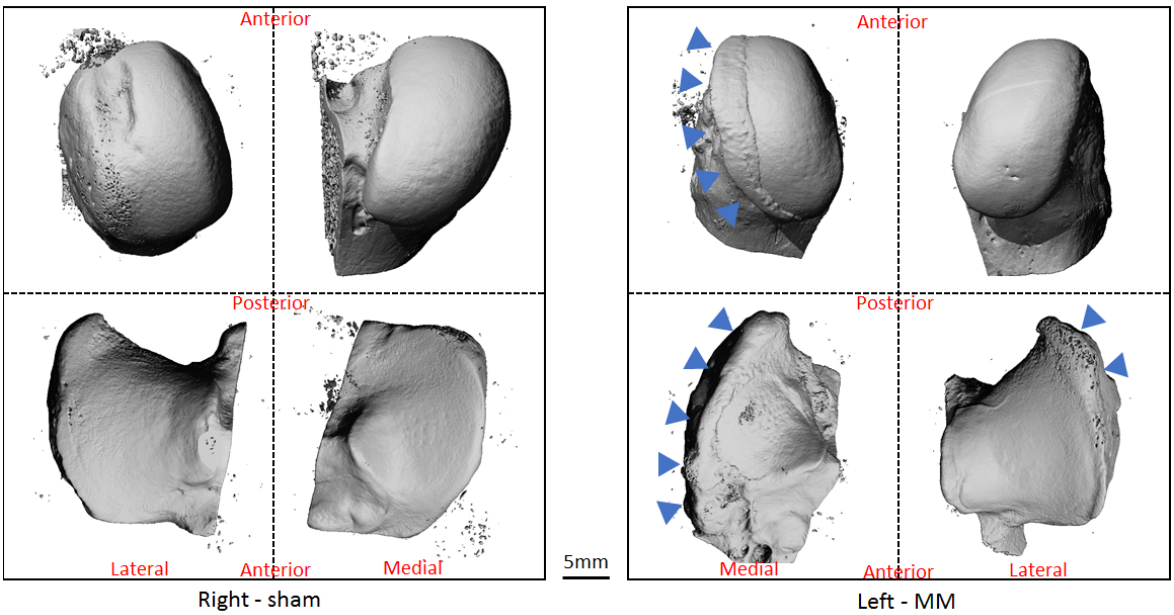

Figure 7. MicroCT 3D renderings of bone only segmentations, same animal as shown in Figure 12. Blue arrows indicate areas where marginal bone changes were qualitatively observed on the medial femoral condyle and medial and lateral condyles of the left MM stifle.

Average 3D articular cartilage thickness, volume, and attenuation were quantified separately for the medial femoral condyle, lateral femoral condyle, medial tibial plateau, and lateral tibial plateau. Data from 24wk post-surgery volumes of interest (VOIs) are shown in Figures 8 and 9.

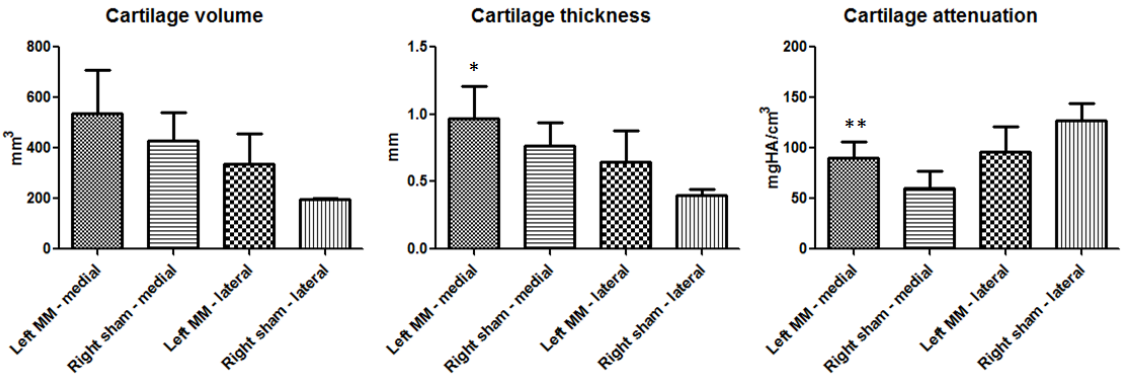

Figure 8. Femoral articular cartilage: Articular cartilage volume, average thickness, and average attenuation were quantified. Left knees underwent medial meniscectomy (MM); right knees = contralateral sham control. For the medial femoral condyles, average cartilage thickness in MM stifles was greater than sham * p=0.0328; average cartilage attenuation for MM was greater than sham ** p=0.0040 (paired t-tests, data shown as mean ± s.d., n=2-3).

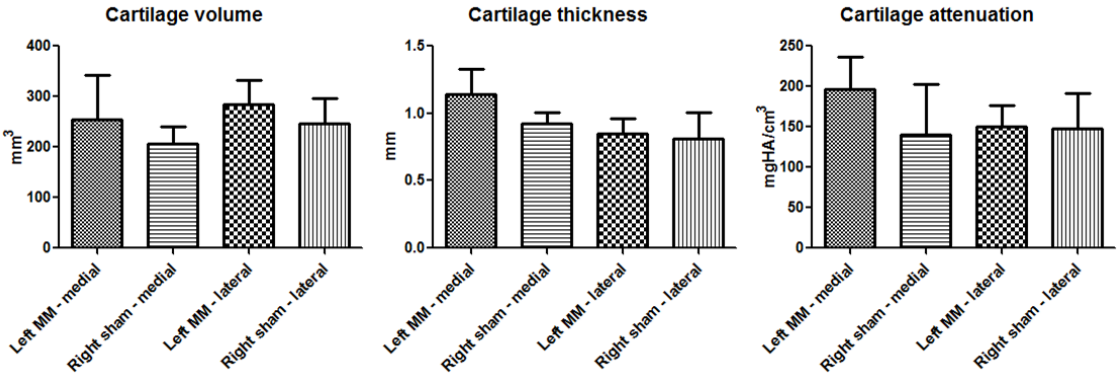

Figure 9. Tibial articular cartilage: Articular cartilage volume, average thickness, and average attenuation were quantified. No significant differences were detected. Data are shown as mean ± s.d., n=3.

Major Task 5: Assess therapeutic efficacy in ovine OA model.

Subtask 1: Surgical and treatment procedures.

- Surgical procedures and subsequent dHACM injections for assessing efficacy of dHACM in the ovine MM model have been performed for a number of animals (surgery dates: 20-JUN-2016, 03-OCT-2016, 26-OCT-2016) with the remaining surgeries scheduled for 14-NOV-2016, and 28-NOV-2016.

Specific Aim 3 Conclusions

The preliminary results indicate increases in cartilage volume, thickness, and attenuation in left (MM) medial joint compartments compared to right (sham) at 12 (shown in previous reports) and 24 weeks post-surgery. Additionally, qualitative bone remodeling in the left (MM) marginal regions of the tibiae and femora have been observed at 12 and 24 weeks post-surgery. These data from preliminary studies suggest that the MM surgical technique in ovine stifle joints induces detectable degenerative changes at 12 weeks post-surgery. Further work to quantify lesion parameters and surface roughness similar to what has been performed for the rat MMT model is ongoing.

Current and Anticipated Problems

There have been a few issues that have resulted in minor adjustments in study timelines, and potential future approaches have been considered, as listed below:

1. *Inflammatory response evaluation in the rat MMT model*: (Specific Aim 1, Major Task 2, Subtask 2; Specific Aim 2, Major Task 3, Subtask 3)

The originally proposed methodology was to extract synovial fluid from rat joints via knee joint lavage technique. However, this technique proved to be inconsistent in acquiring enough fluid to analyze. A potential alternative approach we will explore is to better understand how the dHACM particles, which we have shown becomes embedded in the synovial membrane in the rat MMT model, interact with synoviocytes *in vitro*. Developing *in vitro* systems to assess the inflammatory state of the synovium, release of proteases, inflammatory and anti-inflammatory cytokines, and other factors in response to the presence of micronized dHACM could lead us to a more clear understanding of the mechanisms of OA disease attenuation.

2. *Assessing therapeutic efficacy of dHACM treatment in ovine MM model*: (Specific Aim 3, Major Task 5)

There are two current issues affecting this portion of the project:

IACUC approval for the therapeutic efficacy study in the ovine MM model was extremely delayed. Currently, release of funding for the subaward to SAMMC is still being processed and negotiated. The study has been initiated but is behind schedule. Fortunately, during a site visit to SAMMC, we have formulated a plan to complete the surgeries during this upcoming quarter. With preliminary data suggesting that a 12 week post-MM surgery time point may provide enough detectable damage, exploring a therapeutic window within the 12 week end point could be sufficient without going out to the later 24 week end point (as proposed).

The originally utilized cartilage contrast agent, Hexabrix^{TM}, has been discontinued from production by its manufacturer. This is expected to create delays in evaluating this study's

ovine stifle joint specimens. We are currently testing a cationic contrast agent called CA4+ for these applications, which will involve initial testing and validation in healthy rat joints followed by testing and validation in rat joints after MMT surgeries.

4. KEY RESEARCH ACCOMPLISHMENTS:

1. We determined through work in Specific Aim 2 that intra-articular treatment with dHACM after the onset of OA changes can provide protective effects against disease progression (evaluation of effects at 6 weeks post-MMT surgery when dHACM treatment was administered at 3 wks post-surgery).

2. Evaluation of disease progression at 1 and 3 weeks post-MMT surgery in the rat model (from Specific Aim 1) suggested that degenerative changes in articular cartilage (attenuation and surface roughness) were detectable prior to those of subchondral bone (mineral density and thickness). This addresses a major question in the field on the sequential pathology of post-traumatic OA.

3. Micronized dHACM has been successfully fluorescently labeled at a signal level that is detectable and quantifiable out to 42 days, and this will enable us to identify and spatially locate the dHACM in ongoing histological evaluations.

5. CONCLUSION:

The combined effects of post-traumatic and degenerative OA on active military personnel and veterans are the cause of substantial disability, medical discharge, and long-term costs. Assuming success of this work and further clinical studies to demonstrate safety and effectiveness of the proposed treatment method, the injectable nature of the therapeutic could facilitate accessibility of treatment in the field. Because OA disease progression is accelerated for soldiers who experience high energy combat injuries, one potential course of action would be to administer the therapeutic immediately post-operatively in an effort to prevent the accelerated response and reduce the loss of personnel and costs associated with disability.

An important consideration for injectable OA therapeutics is the ability to be retained in the joint and therefore sustain beneficial effects and minimize the frequency of injections. This work will provide necessary preclinical data including safety and immune response, retention, and long-term effectiveness of the particle injections. If successful, this project will present a readily translatable, low risk, minimally invasive treatment method that modifies OA disease progression in a clinically relevant model, leading to human clinical trials as the clear next step.

Much progress has been made thus far on the study aims. For the small animal studies, two particle size distributions of dHACM were assessed for their therapeutic effects after MMT, and the standard micronized formulation of dHACM (AmnioFix®) resulted in better preservation of articular cartilage PG content after 1 and 3 weeks. Reducing the particle size (RPS AmnioFix®) did not further attenuate OA disease progression compared to the standard formulation and resulted in higher average cartilage thickness at 3 weeks. Surface roughness was utilized effectively as a measure

for cartilage degenerative changes at 1 and 3 weeks post-MMT. Effects of single early, single delayed, and multiple dHACM intra-articular treatments were assessed in the rat MMT model, and the single delayed injection (emulating treatment after disease progression has begun) provided a protective effect against degradative changes in the joint. dHACM particles have been successfully labeled with a fluorophore, which will allow us to further assess the retention profiles of two particle size distributions in the rat MMT model. Work is ongoing to more mechanistically understand the response of joint cells and tissues to dHACM.

Characterization of the large animal model (ovine MM) has indicated that degenerative changes in cartilage and marginal osteophytes are seen at 12 weeks and 24 weeks post-surgery. One critical focus area that remains to be completed is assessing the effects of dHACM in the ovine MM model of PTOA. This therapeutic efficacy study is ongoing, and all the needed dHACM material has been received by SAMMC to complete the remaining surgeries and injections. These are expected to be completed during the upcoming quarter.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

Presentations:

Type	**Description**
Conference presentation / poster	Reece, D.S., Lin, A., Willett, N.J., Guldberg, R.E. Early Joint Changes in a Pre-Clinical Osteoarthritis Model. Military Health System Research Symposium (MHSRS), August 2016.

7. INVENTIONS, PATENTS AND LICENSES: Nothing to report.

8. REPORTABLE OUTCOMES: These studies have resulted in creation of a research tool based on microCT scans to measure two novel 3D metrics for joint degeneration: surface roughness (based on differences with model surfaces) and exposed bone area. It has been tested and validated for our preclinical rat model of PTOA and will be tested and validated in the ovine model. This algorithm may provide benefit in the investigation of early effects of potential therapeutics.

9. OTHER ACHIEVEMENTS: Nothing to report.